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<b>(54) Title:</b> IMPROVED PROCESS FOR THE PRODUCTION OF SEMI-SYNTHETIC CEPHALOSPORINS VIA EXPANDASE ACTIVITY ON PENICILLIN G  <b>(57) Abstract</b>  An overall process for the preparation and recovery of 7-aminodesacetoxycephalosporanic acid (7-ADCA) via enzymatic ring expansion activity on penicillin G, using a modified expandase enzyme.		

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IMPROVED PROCESS FOR THE PRODUCTION OF  
SEMI-SYNTHETIC CEPHALOSPORINS  
VIA EXPANDASE ACTIVITY ON PENICILLIN G

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Field of the invention and brief description of the prior art

The present invention concerns new mutant expandases, to be used in a biosynthetic process for preparation and recovery  
10 of 7-aminodesacetoxycephalosporanic acid (7-ADCA), one of the key intermediates used in the preparation of semi-synthetic cephalosporins (SSC's).

$\beta$ -Lactam antibiotics constitute the most important group of antibiotic compounds, with a long history of clinical use.  
15 Among this group, the prominent ones are the penicillins and cephalosporins. These compounds are naturally produced by the filamentous fungi Penicillium chrysogenum and Acremonium chrysogenum, respectively.

As a result of classical strain improvement techniques, the  
20 production levels of the antibiotics in Penicillium chrysogenum and Acremonium chrysogenum have increased dramatically over the past decades. With the increasing knowledge of the biosynthetic pathways leading to penicillins and cephalosporins, and the advent of recombinant DNA technology, new tools for the  
25 improvement of production strains and for the in vivo derivatization of the compounds have become available.

Most enzymes involved in  $\beta$ -lactam biosynthesis have been identified and their corresponding genes been cloned, as can be found in Ingolia and Queener, Med. Res. Rev. 9 (1989), 245-264  
30 (biosynthesis route and enzymes), and Aharonowitz, Cohen, and Martin, Ann. Rev. Microbiol. 46 (1992), 461-495 (gene cloning).

The first two steps in the biosynthesis of penicillin in P. chrysogenum are the condensation of the three amino acids L-5-amino-5-carboxypentanoic acid (L- $\alpha$ -aminoadipic acid) (A),

L-cysteine (C) and L-valine (V) into the tripeptide LLD-ACV, followed by cyclization of this tripeptide to form isopenicillin N. This compound contains the typical  $\beta$ -lactam structure.

The third step involves the exchange of the hydrophilic side chain of L-5-amino-5-carboxypentanoic acid by a hydrophobic side chain by the action of the enzyme acyltransferase (AT). The enzymatic exchange reaction mediated by AT takes place inside a cellular organelle, the microbody, as has been described in EP-A-0448180.

Cephalosporins are much more expensive than penicillins. One reason is that some cephalosporins (e.g. cephalixin) are made from penicillins by a number of chemical conversions. Another reason is that, so far, only cephalosporins with a n-aminoadipoyl-carboxypentanoyl side chain could be fermented. Cephalosporin C, by far the most important starting material in this respect, is very soluble in water at any pH, thus implying lengthy and costly isolation processes using cumbersome and expensive column technology. Cephalosporin C obtained in this way has to be converted into therapeutically used cephalosporins by a number of chemical and enzymatic conversions.

The methods currently favoured in industry to prepare the intermediate 7-ADCA involve complex chemical steps leading to the expansion and derivatization of penicillin G. One of the necessary chemical steps to produce 7-ADCA involves the expansion of the 5-membered penicillin ring structure to a 6-membered cephalosporin ring structure (see for instance US 4,003,894). This complex chemical processing is both expensive and noxious to the environment.

Consequently, there is a great desire to replace such chemical processes with enzymatical reactions such as enzymatic catalysis, preferably during fermentation. A key to the replacement of the chemical expansion process by a biological process is the central enzyme in the cephalosporin biosynthetic pathway, desacetoxycephalosporin C synthase, or expandase.

The expandase enzyme from the bacterium Streptomyces clavuligerus was found to carry out, in some cases, penicillin ring expansions. When introduced into P. chrysogenum, it can

convert the penicillin ring structure into the cephalosporin ring structure, as described in Cantwell *et al.*, Proc. R. Soc. Lond. B. 248 (1992), 283-289. The expandase enzyme has been well characterized (EP-A-0366354) both biochemically and functionally, as has its corresponding gene. Both physical maps of the cefE gene (EP-A-0341892), DNA sequence and transformation studies in P. chrysogenum with cefE have been described.

Other sources for a ring expansion enzyme are the bacteria Nocardia lactamdurans (formerly Streptomyces lactamdurans) and Lysobacter lactamgenus. Both the biochemical properties of the enzyme and the DNA sequence of the gene have been described (Cortés *et al.*, J. Gen. Microbiol. 133 (1987), 3165-3174; and Coque *et al.*, Mol. Gen. Genet. 236 (1993), 453-458, respectively).

Since the expandase catalyses the expansion of the 5-membered thiazolidine ring of penicillin N to the 6-membered dihydrothiazine ring of DAOC this enzyme would be of course a logical candidate to replace the ring expansion steps of the chemical process. Unfortunately, the enzyme works on the penicillin N intermediate of the cephalosporin biosynthetic pathway, but not efficiently on the readily available inexpensive penicillins as produced by P. chrysogenum, like penicillin V or penicillin G. Penicillin N is commercially not available and even when expanded, the D- $\alpha$ -aminoadipoyl side chain cannot be easily removed by penicillin acylases.

It has recently been found that the expandase enzyme is capable of expanding penicillins with particular side chains to the corresponding 7-ADCA derivative. This feature of the expandase has been exploited in the technology as disclosed in EP-A-0532341, WO95/04148 and WO95/04149. In these disclosures the conventional chemical conversion of penicillin G to 7-ADCA has been replaced by the in vivo conversion of certain 6-aminopenicillanic acid (6-APA) derivatives in recombinant Penicillium chrysogenum strains containing an expandase gene.

More particularly, EP-A-0532341 teaches the in vivo use of the expandase enzyme in P. chrysogenum, in combination with a 5-carboxypentanoyl side chain as a feedstock, which is a



substrate for the acyltransferase enzyme in P. chrysogenum. This leads to the formation of 5-carboxypentanoyl-6-APA, which is converted by an expandase enzyme introduced into the P. chrysogenum strain to yield 5-carboxypentanoyl-7-ADCA.  
5 Finally, the removal of the 5-carboxypentanoyl side chain is suggested, yielding 7-ADCA as a final product.

In WO95/04148 and WO95/04149 it has been disclosed that 3'-carboxymethylthiopropionic acid and 3,3'-thiodipropionic acid, respectively were found to be substrates for the expandase,  
10 yielding 2-(carboxyethylthio)acetyl- and 3-(carboxymethylthio)propionyl-7-ADCA.

However, the process of the present invention provides more advantages, because of the high penG biosynthetic capacity of penicillin producing strains and the more favourable process of  
15 extraction of phenylacetyl-7-ADCA acid. Furthermore the phenylacetyl side chain of phenylacetyl-7-ADCA is very amenable to enzymatic cleavage, by penicillin G amidases produced by several types of microorganisms yielding 7-ADCA, for instance penG acylase as disclosed in EP-A-0453047.

20 Various publications have reported the expandase not to accept penicillin G or penicillin V as a substrate for expansion (Baldwin & Abraham (1988), Natural Product Reports, 5(2), p.129-145; Maeda et al. (1995), Enzyme and Microbial Technology, 17, 231-234; Crawford et al. (1995), Bio/technology, 13, p.58-61).  
25 In contrast to those observations one report mentions an activity of expandase on penicillin G in vitro (Baldwin et al. (1994), Proceedings of the 7<sup>th</sup> International Symposium on the Genetics of Industrial Microorganisms, Abstract P.262).

Recently, the structure of the isopenicillin N synthase (IPNS) enzyme of A. nidulans has been determined (Roach (1995),  
30 Nature, 375, p700-704). IPNS and expandase belong to the same family of oxidase enzymes. They share biochemical characteristics and, on the basis of sequence homologies, it has been proposed that structural similarities exist between the two  
35 enzymes (Roach et al., supra; Cooper (1993), Bioorganic Med. Chem. 1, p1-17).





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The mechanism of IPNS activity has been described in several reports (see for example: Blackburn et al. (1995), Biochemistry 34, p7548-7562). It is proposed, from an analysis of the chemistry catalysed by IPNS, that the cysteinyl thiol  
5 group of ACV must bind to the ferrous ion at the active site in the enzyme-substrate complex. Given this implicit attachment point between the substrate and the enzyme a large number of conformationally distinct binding modes can be distinguished given the crystallographically determined constraints of the  
10 active site. It is therefore not obvious how the aminoadipoyl side-chain of ACV binds to A. nidulans IPNS (aIPNS) and, by inference, the mode of binding of penicillin N to expandase is even less apparent.

15

#### Figures

Figure 1: Alignment of expandase from Streptomyces clavuligerus versus isopenicillin N synthase from Aspergillus  
20 nidulans.



### Summary of the invention

The present invention provides new mutant expandase enzymes, especially suitable for the preparation and recovery of 7-aminodesacetoxycephalosporanic acid (7-ADCA) by:

- a) transforming a Penicillium chrysogenum strain with a modified expandase gene, under the transcriptional and translational regulation of fungal expression signals;
- b) fermenting said strain in a culture medium and adding to said culture medium phenylacetic acid or a salt or ester thereof suitable to yield penicillin G, which is expanded to form phenylacetyl-7-ADCA;
- c) recovering the phenylacetyl-7-ADCA from the fermentation broth;
- d) deacylating phenylacetyl-7-ADCA; and
- e) recovering the crystalline 7-ADCA.

Preferably, step (e) is a filtration step.

Preferably, phenylacetyl-7-ADCA is recovered from the fermentation broth by extracting the broth filtrate with an organic solvent immiscible with water at a pH of lower than about 4.5 and back-extracting the same with water at a pH between 4 and 10.

Moreover, the DNA encoding modified expandase and a recombinant DNA vector comprising the same, functionally linked to the transcriptional and translational control elements of a fungal gene, for instance Aspergillus nidulans godA gene, and the Aspergillus niger glcA gene and host cells transformed with the same, are provided.

### Detailed description of the invention

The present invention concerns the use of functional gene constructs encoding modified expandase enzyme in P. chrysogenum for the in vivo expansion of the penicillin G ring structure to form the phenylacetyl acid derivative of a key intermediate in the cephalosporin biosynthesis, 7-aminodesacetoxycephalosporanic



acid, or 7-ADCA. This derivative has a chemical composition so as to allow efficient solvent extraction, thus providing an economically attractive recovery process.

Modification of the expandase gene is directed at producing  
5 mutants which best expand penicillin G in in vitro and/or in vivo context where other penicillins such as penicillin N and isopenicillin N can act as competing substrates. This is an important feature of the invention given the observation of significant amounts of penicillin N being produced by P. chrysogenum  
10 and the knowledge that penicillin N is a significantly better substrate than penicillin G for the wildtype expandase (Alvi et al., J. Antibiotics, 338 (1995)). By transforming P. chrysogenum with such targeted mutants of expandase, novel P. chrysogenum strains can be obtained which  
15 have an improved capacity for the production of phenylacetyl-7-ADCA. Mutant expandase enzymes suitable to expand penicillin G have been screened as follows:

- i) Identifying amino acid residues of expandase involved in penicillin side-chain recognition and binding;
- 20 ii) Construction of modified genes encoding mutant expandase proteins in a form easily purifiable as fusions to maltose-binding protein (MBP);
- iii) Characterising the binding and catalytic properties of said mutant expandases towards penicillins specifically those  
25 with phenylacetyl and  $\alpha$ -aminoadipoyl side-chains.

This allows those skilled in the art to construct strains with improved phenylacetyl-7-ADCA production capacity for a 7-ADCA process as described by the non-prepublished international application PCT/EP 96/02434, filing date June 3, 1996.  
30 Transformation of P. chrysogenum can, in principle, be achieved by different means of DNA delivery, like PEG-Ca mediated protoplast uptake, electroporation or particle gun techniques, and selection of transformants. See for example Van den Hondel en Punt, Gene Transfer and Vector Development for Filamentous  
35 Fungi, in: Applied Molecular Genetics of Fungi (Peberdy, Laten, Ogden, Bennett, eds.), Cambridge University Press (1991). The application of dominant and non-dominant selection markers has

been described (Van den Hondel, supra). Selection markers of both homologous (P. chrysogenum derived) and heterologous (non-P. chrysogenum derived) origin have been described (Gouka et al., J. Biotechnol. 20 (1991), 189-200).

5 In summary, the present invention teaches how a modified expandase gene introduced into P. chrysogenum can be used to improve the yield of phenylacetyl-7-ADCA resulting from the in vivo ring expansion of penicillin G by a mutant expandase.

10 Methods for the recovery of phenylacetyl-7-ADCA from the fermentation broth and for the conversion to 7-ADCA and for the recovery of 7-ADCA have been disclosed in the non-pre-published application No. PCT/EP 96/024340 mentioned above.

The following examples are offered by way of illustration and not by way of limitation. The overall approach entails i)  
15 identification of residues of expandase involved in penicillin side-chain recognition and binding specificity, ii) construction of mutant expandase proteins in a form easily purified as fusion proteins to maltose-binding-protein (MBP), iii) assessment of activity towards different penicillins as penicillin N and  
20 penicillin G following expression in E. coli and purification.

#### Example 1

25 Identification of residues involved in substrate side-chain binding

Central to the invention is the proposal that, in the case of aIPNS (IPNS from Aspergillus nidulans), upon ACV binding, the L- $\alpha$ -aminoadipoyl side chain of ACV displaces the C-terminal tail  
30 of the enzyme (glutamine 330, threonine 331 and a number of preceding residues) by virtue of the similarity between the L- $\alpha$ -aminoadipoyl side chain of ACV and the C-terminal dipeptide in steric and electronic terms. Comparison of the C-terminal tail and ACV reveals the similarity between the L- $\alpha$ -aminoadipoyl  
35 side-chain of ACV and the glutamyl-threonine end of the tail; specifically the carboxylates in both cases are functionally homologous. In the resting state of the aIPNS enzyme, the

carboxylate of the C-terminal threonine residue is in a position to form hydrogen-bonds to arginine 87, polar contacts to serine 183 and hydrophobic contacts with valine 185 and phenylalanine 285. Upon ACV binding to aIPNS it is proposed that serine 183 and arginine 87 can interact with the L- $\alpha$ -aminoadipoyl side-chain carboxylate by polar effects and that hydrophobic contacts can be made between the methylene groups and amide of the side chain to valine 185 and phenylalanine 285. The sequence similarities of expandase and aIPNS along with common spectroscopic properties and similar chemistries suggest that the D- $\alpha$ -aminoadipoyl side chain of the substrate penicillin N binds in a similar fashion to expandase as does the L- $\alpha$ -aminoadipoyl side chain of ACV to aIPNS.

At the heart of the invention is the proposal that the  $\alpha$ -aminoaminoadipoyl side chain of penicillin N will be bound by amino acid residues of expandase that are structurally analogous in the context of the 3D protein structure (and can be identified by sequence homology analysis homology analysis) to the amino acid residues of aIPNS involved in binding the  $\alpha$ -aminoadipoyl side chain of ACV. In addition to these residues other residues that can be similarly identified by examination of the aIPNS crystal structure using the aforementioned substrate-binding model.

The amino acid sequence of Streptomyces clavuligerus expandase is shown in figure 1. The numbers indicate the position of an amino acid in the sequence and will be used as an indication for the amino acid position in the description of amino acid changes. Regarding the corresponding amino acid changes in expandase which are homologous with Streptomyces clavuligerus expandase, the skilled person will understand that the Streptomyces clavuligerus amino acid position used herein refer to the corresponding conserved amino acids in the amino acid sequence of these related enzymes and not necessarily to their amino acid positions in those enzymes. It is also to be understood that these corresponding conserved amino acids are not necessarily identical to those of Streptomyces clavuligerus expandase. Corresponding position with respect to Streptomyces

clavuligerus can be obtained by standard alignment procedures as are known by persons skilled in the art.

Residues of Streptomyces clavuligerus expandase so identified include, but are not restricted to arginine 74  
5 (homologous to arginine 87 of aIPNS), cysteine 155 (homologous to serine 183 of aIPNS), proline 157 (homologous to valine 185 of aIPNS), leucine 159 (homologous to isoleucine 187 of aIPNS), phenylalanine 264 (homologous to phenylalanine 285 of aIPNS), isoleucine 298 (homologous to leucine 317 of aIPNS), tyrosine  
10 302 (homologous to leucine 321 of aIPNS), arginine 306 (homologous to isoleucine 325 of aIPNS) and arginine 266 (homologous to asparagine 287 of aIPNS). Mutation of these residues individually or in combination will alter the relative binding of penicillin N and penicillin G to expandase in the  
15 ground state and subsequent intermediates and transition states for the expansion of these penicillins to DAOC and phenylacetyldesacetoxycephalosporin, respectively. Mutations at the aforementioned positions of expandase will increase the expansion of penicillin G, decrease the expansion of penicillin  
20 N and/or increase the relative ratio of penicillin G to other penicillin expansion in a competitive scenario.

Many benefits accrue to a process involving in vivo expansion of penicillin G to phenylacetyl-7-ADCA as described in the non-pre-published application No. PCT/EP 96/02434, mentioned above.  
25 The wild type expandase accepts penicillin N as its normal substrate with conflicting reports concerning the acceptance of penicillin G as a substrate. In order to improve penicillin G as an isolated substrate it is necessary to improve  $v_{max}$  and, in a context where the concentration of penicillin G is non-saturating, to lower the  $K_m$ . This is not only the case when  
30 penicillin G is an isolated substrate but also when penicillin G is a substrate in the presence of other penicillins, in the first place penicillin N but also isopenicillin N. Thus, for example, in the microbody location of P. chrysogenum cefE  
35 transformants precursed with phenylacetic acid, the expandase enzyme can act on penicillin N in competition with penicillin G.



The relative and absolute amounts of each penicillin expanded depend on the ratio of the individual rates which can be broken down into an equation of the form:

$$\begin{array}{l} \text{rate of expansion of penicillin G} = V^G = V_{\max}^G [G] K_M^N \\ \text{-----} \\ \text{rate of expansion of penicillin N} = V^N = V_{\max}^N [N] K_M^G \end{array}$$

where  $V_{\max}^G$  and  $V_{\max}^N$  correspond to the maximum enzyme velocities,  $K_M^N$  and  $K_M^G$  are the Michaelis constants, and  $[G]$  and  $[N]$  are the concentrations of penicillin G and penicillin N respectively. Mutations at positions of the expandase listed below which result in an increase of the ratio of  $V^G : V^N$  are part of the invention. The specificity changes required can result from any single or multiple mutant that has values of  $V_{\max}$  and/or  $K_M$  for either or both substrates altered in any way such as to increase the ratio of  $V^G : V^N$  in vitro or the relative yield of phenylacetyl-7-ADCA compared to DAOC from a phenylacetate derivative precursed fermentation of a strain of P. chrysogenum transformed with the mutant cefE gene. In the first instance improvement in the competition between penicillin G and penicillin N will occur but also between penicillin G and isopenicillin N such as to improve the yield of phenylacetyl-7-ADCA.

## Example 2

### Construction of mutants

a) General techniques for gene cloning and manipulation are well described in Sambrook et al., Molecular Cloning, a laboratory Manual, Cold Spring Harbour, USA (1989).

b) Vector construction:

A chloramphenicol gene cartridge (Pharmacia, HindIII cartridge) is blunt-ended by treatment with Klenow fragment and four dNTPs and ligated to ScaI linearised pMAL-c2 (New England Biolabs) then used to transform competent E. coli cells to

chloramphenicol resistance. Resultant clones are restriction mapped with NcoI, ApaI and other enzymes and a plasmid in which the direction of transcription of the chloramphenicol resistance gene is the same as that of the malE gene is designated pAJL100.

5 A linker prepared by self-annealing of the palindromic oligonucleotide, AL5 (5'TAC CGA ATT CCG3') is ligated to NdeI linearised pNM88 (Morgan et al. (1994), Bioorg. Med. Lett. 4, p1595-1600) and the resulting ligation is sanitised by digestion with NdeI before being used to transform E. coli. Resultant

10 clones are restriction mapped by digestion with EcoRI, SalI and other enzymes and a plasmid giving the anticipated restriction pattern is designated pAJL103. The Streptomyces clavuligerus cefE gene is subcloned as an EcoRI/SalI fragment from pAJL103 into the corresponding polylinker sites of pAJL100 giving

15 pAJL104 which is characterised by restriction mapping with ApaI, BglI, EcoRI, ScaI and other enzymes.

c) Expression in E. coli:

Expression of the malE-cefE gene fusion is achieved by induction

20 of a culture of E. coli NM554[pAJL104] with IPTG. Induced cells are lysed by treatment with lysozyme followed by sonication and released protein is quantitated by Bradford assay. Purification of the MBP-expandase fusion protein is achieved by application of crude cellular lysates to an amylose column, washing and

25 subsequent elution with buffer containing maltose to give a protein of approximately 77 kDa molecular weight as assessed by SDS-PAGE against molecular weight standards. MBP-DAOCS fusion is assayed for bioactivity using penicillin N as a substrate using reverse-phase HPLC and hole-plate bioassay against super

30 sensitive E. coli in the presence of penicillinase. Active protein results in zones of lysis on bioassay plates and a new product with the same retention time as synthetic DAOC on reverse phase HPLC with a variety of buffer conditions and monitoring at 220 and 254nm. MBP-DAOCS fusion is assayed for

35 bioactivity using penicillin G as a substrate using reverse-phase HPLC and 500 MHz <sup>1</sup>H NMR spectroscopy with confirmatory sample spiking in both cases.

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## d) Mutagenesis of expandase gene:

Uracil-containing single-stranded pAJL104 DNA is obtained from cultures of E. coli BW313 [pAJL104] following superinfection with helper phage M13 KO7. This uracil-containing single-stranded pAJL104 DNA is used in mutagenesis experiments in which 5'-phosphorylated synthetic oligonucleotides are annealed and extended in the presence of T7 DNA polymerase, T4 DNA ligase, dNTPs and ATP. Following second-strand synthesis the reactions are used to transform competent E. coli NM554 or XL1 Blue to chloramphenicol resistance. Resultant clones are restriction mapped or sequenced to confirm the presence of the desired mutation. As examples of mutants constructed in this manner:

15 Mutation of argine 74 (arginine 87 in aIPNS):

The R74F mutation is introduced using the phosphorylated primer: 5'ACC ATG TTT CGC GGC TTC ACC3'. Resultant clones are mapped by digestion with SacII, NcoI and other enzymes and the plasmid giving the anticipated fragments is designated pAJL211. The loss of a SacII site in pAJL211 relative to pAJL104 confirms the introduction of the TTT codon encoding phenylalanine in pAJL211. The R74I, R74M and R74Q mutations are introduced in the following way. XhoI and SpeI sites flanking the R74 coding region in pAJL104 were introduced using two phosphorylated primers:

5'GAA GCG CGC CGT CAC TAG TCC CGT CCC CAC CAT G3' and  
5'CTT CAC CGG GCT CGA GTC GGA GAG C3'.

The resulting plasmid is designated pGEN50.

The R74M mutation is then introduced by subcloning a synthetic cassette prepared by annealing two oligonucleotides:

5'CTA GTC CGG TAC CGA CCA TGA TGA GGG GAT TCA CTG GTC3' and  
5'TCG AGA CCA GTG AAT CCC CTC ATC ATG GTC GGT ACC GGA3'.

The introduction of the cassette was indicated by the presence of a new KpnI site in the resultant plasmid which was designated pGEN51.

The R74Q mutation is then introduced by subcloning a synthetic cassette prepared by annealing two oligonucleotides:

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5'CTA GTC CGG TAC CGA CCA TGC CAA GGG GAT TCA CTG GTC3' and  
5'TCG AGA CCA GTG AAT CCC CTT TGC ATG GTC GGT ACC GGA3'.

The introduction of the cassette was indicated by the presence  
of a new KpnI site in the resultant plasmid which was designated  
5 pGEN52.

The R74Q mutation is then introduced by subcloning a synthetic  
cassette prepared by annealing two oligonucleotides:

5'CTA GTC CGG TAC CGA CCA TGA TCA GGG GAT TCA CTG GTC3' and  
5'TCG AGA CCA GTG AAT CCC CTG ATC ATG GTC GGT ACC GGA3'.

10 The introduction of the cassette was indicated by the presence  
of a new KpnI site in the resultant plasmid which was designated  
pGEN53.

The sequence of the clones in the region of the residue 74  
coding sequence was verified by sequence analysis using a  
15 primer:

5'GAG CTG AAG TCG GCC AAG3'.

Mutation of cysteine 155 (serine 183 in aIPNS):

20 The C155L mutation is introduced using the phosphorylated  
primer:

5'GAG GCC TTC CTC GAC CTC GAG CCG CTG CTG CGG3'. Resultant  
clones are mapped by digestion with XhoI, NcoI and other enzymes  
and the plasmid giving the anticipated fragments is designated  
25 pAJL201. The XhoI site in pAJL201 confirms the presence of the  
CTC codon encoding leucine.

The C155A mutation is introduced using the phosphorylated  
primer:

5'GTC GAG GCC TTC CTC GAC GCT GAG CCG CTG CTG CGG TTC CG3'.  
30 Resultant clones are mapped by digestion with ApaI, BpuI 1021  
and ScaI and the plasmid giving the anticipated fragments is  
designated pGEN70.

The C155V mutation is introduced using the phosphorylated  
primer:

35 5'GTC GAG GCC TTC CTC GAC GTC GAG CCG CTG CTG CGG TTC CG3'.  
Resultant clones are mapped by digestion with AatII, EcoRI and

- 15 -

other enzymes and the plasmid giving the anticipated fragments is designated pJC14.

The sequence of the clones in the residue 155 coding region was verified by sequence analysis using a primer:

5' AGC GGA TCT GGA CCC AGT3'.

Mutation of proline 157 (valine 185 in aIPNS):

The P157G and P157A mutations are introduced using the phosphorylated primer mixture:

5' GCC TTC CTT GAC TGC GAA NNN CTT CTC CGT TTT CGC TAC TTC CCG3'

where N represents a mixture of G, A, T and C. Resultant clones are mapped by digestion with XmnI, NcoI and other enzymes and plasmids having the extra XmnI site are analysed further by digestion with StuI and Eco47III. Plasmids having an added StuI site are designated pAJL232; the added XmnI and StuI sites confirm the presence of a GGC codon encoding glycine. Plasmids having an added Eco47III site are designated pAJL233; the added XmnI and Eco47III sites confirm the presence of a GCG codon encoding alanine.

Mutation of arginine 266 (asparagine 287 in aIPNS):

The R266N mutation is introduced using the phosphorylated primer:

5' CC TCC AGT GTG TTC TTT TTA AAT CCC AAC GCG GAC TTC3'.

Resultant clones are mapped by digestion with DraI, NcoI and other enzymes and the plasmid giving the anticipated fragments is designated pMJS1. The introduction of a fifth DraI site confirms the presence of an AAT codon encoding asparagine.

The R266Q mutation is introduced using the phosphorylated primer:

5' AGT GTG TTC TTC CTG CAG CCC AAC GCG GAC3'. Resultant clones are mapped by digestion with PstI, EcoRI and other enzymes and the plasmid giving the anticipated fragments is designated pMJS2. The introduction of a PstI site confirms the mutation.

- 16 -

The R266M mutation is introduced using the phosphorylated primer:

5' GCG GGC AGC AGC CGC ACG AGC TCT GTG TTC TTC CTC ATG CCC AAC  
GCG GAC TTC 3'. Resultant clones are mapped by digestion with  
5 SacI, EcoRI and other enzymes and the plasmid giving the  
anticipated fragments is designated pMJS3. The introduction of  
a SacI site confirms the mutation.

### 10 Example 3

#### Characterisation of mutants

The mutants are characterised by HPLC analysis of expansion  
reactions of penicillin N and penicillin G both individually and  
15 in mixtures of differing proportions and amounts. DAOC and  
phenylacetyldeacetoxycephalosporin are identified by retention  
times using several different buffers and elution conditions  
with monitoring at 220 and 254nm, and by confirmatory sample  
spiking. 500 MHz <sup>1</sup>H NMR analysis is also used to confirm the  
20 nature of products by comparison with synthetic standards. As  
examples the following data are obtained:

The R266N mutant is found to expand penicillin N and penicillin  
G less well than the wild-type expandase. In mixtures comprising  
penicillin N and G, the R266N mutant displays a higher ratio of  
25  $v_G : v_N$  than the wild-type expandase.

The R74F mutant is found to expand penicillin N poorly and peni-  
cillin G very poorly. The R74I mutant shows slightly lowered  
activity on penicillin N, this activity is greatly reduced in  
the presense of penicillin G. The C155L mutant is found to  
30 expand penicillin N well and penicillin G poorly relative to the  
wild-type expandase. In mixtures containing penicillin N and  
penicillin G, penicillin N expansion is inhibited by penicillin  
G. The R74I and C155L mutants both show increased affinity for  
penicillin G relative to penicillin N, the former more so than  
35 the latter.

These examples demonstrate that mutations in these  
positions affect the ratio of  $v_G : v_N$ , the turnover rate of

penicillin N and the affinity for penicillin G in competition with aminoadipoyl penicillins.

## CLAIMS

5

1. A mutant expandase comprising:

- a) a substitution at one or more selected sites corresponding to a residue position selected from the group consisting of arginine 74, cysteine 155, proline 157, leucine 159, phenylalanine 264, isoleucine 298, tyrosine 302, arginine 306 and arginine 266,
- b) related to said wildtype expandase, an altered substrate specificity.

15

2. A mutant expandase according to claim 1 comprising one or more mutations selected from the group consisting of (a) R74F, R74M, R74Q, R74I; (b) C155L, C155A, C155V; (c) P157G; (d) P157A; (e) R266N; (f) R266Q; and (g) R266M.

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3. A modified expandase gene encoding the expandase mutants as defined in claim 1 or 2.

4. An expression vector which comprises a modified expandase gene as defined in claim 3.

25

5. A microorganism host strain transformed with an expression vector as defined in claim 4.

6. An improved process for the preparation and recovery of 7-aminodesacetoxycephalosporanic acid (7-ADCA) by:

- a) transforming a Penicillium chrysogenum strain with a modified expandase gene as defined in claim 3, under the transcriptional and translational regulation of fungal expression signals;

35

b) fermenting said strain in a culture medium and adding to said culture medium phenylacetic acid or a salt or ester thereof suitable to yield penicillin G, which is expanded to form phenylacetyl-7-ADCA;



- c) recovering the phenylacetyl-7-ADCA from the fermentation broth;
- d) deacylating phenylacetyl-7-ADCA; and
- e) recovering the crystalline 7-ADCA.

5

7. A process according to claim 6, wherein step (e) is a filtration step.

8. A process according to claim 6 or 7, wherein step (c) is a filtration step, and by extracting the broth filtrate with an organic solvent immiscible with water at a pH of lower than about 4.5 and back-extracting the same with water at a pH between 4 and 10.

9. A process according to anyone of the claims 6, 7 or 8 wherein the expandase gene is derived from Streptomyces clavuligerus, Nocardia lactamdurans or Lysobacter lactamgerus.

Figure 1

```

---1-----+...10-----+...20...+...30...+...40...+...50...+...60...+...70-
---M-----DTVPTFSLAEL-----QQGLHQDEFRRCLRDKGLFYLTDCGLTDTELKSAKDIVIDFFEHGSEAEKRAVTSVP--
* * * * *
---MGSVSKANVPKIDVSPFGDDQAAMRVAQQIDAASRD TGFFYAVNHGI--NVQRLSQKTKEFHMSITPEEKWDLAIRAYN
---1...+...10...+...20...+...30...+...40...+...50...+...60...+...70...+...
-----+...80...+...90...+...100...+...110...+...120...+...130...+...
-----PTMRRGFTGLESESTAQITNTGSDYSYCMYSGMTADN-----LFPSGD-----FERIWTQYFDRQYTASRAVAREVLRAT
* * * * *
KEHQDQVRAGYLSIPGKKAVESFCYLNPNFTPDHPRIQAKTPTHEVNVWPDETKHPGFQDFAEQYYWDVFGLSALLKGYALAL
80...+...90...+...100...+...110...+...120...+...130...+...140...+...150...+...160...
...+...150...+...160...+...170...+...180...+...190...+...200...+...210...+...
GTEPD--GGV--EAFLDCEPLLRFRYPFPQVPEHRS--EEQPLRMAPHYDLSMVTLIQQTPCANGFVSLQAEVGGAFDLPYR
* * * * *
GKEENFFARHFKPDDTLASVVLIRYPYLDYPPEAAIKTAADGTKLSFEWHEDVSLITVLYQSNVQN---LQVETAAGYQDIEAD
+...170...+...180...+...190...+...200...+...210...+...220...+...230...+...240...+
.220...+...230...+...240...+...250...+...260...+...270...+...280...+...290...+...300.
PDAVLVFCGAIATLVTTGGQVKAPRRHHVAAPRRDQIAGSSRTSSVFFLRPNADFTFSVPLARECGFDVSLDGETATFQDWIGGNVY
* * * * *
DTGYLINCGSYMAHLTNYYKAPIHRVK-----WVNAERQSLPFFVNLGYDSVIDPDPREPNGKS--DREPLSYGDYLONGLV
...250...+...260...+...270...+...280...+...290...+...300...+...310...+...320
...+...310
NIRRTSKA
SLINKNGQT
...+...330

```

DAOCS-S.clav  
 Identity:  
 IPNS-E.nidul

DAOCS-S.clav  
 Identity:  
 IPNS-E.nidul

DAOCS-S.clav  
 Identity:  
 IPNS-E.nidul

DAOCS-S.clav  
 Identity:  
 IPNS-E.nidul

DAOCS-S.clav  
 Identity:  
 IPNS-E.nidul

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/52, 9/00, C12P 35/00, C12N 1/21</b>		<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 97/20053</b>
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<b>(21) International Application Number:</b> PCT/EP96/05358 <b>(22) International Filing Date:</b> 27 November 1996 (27.11.96)  <b>(30) Priority Data:</b> 95203259.7                      27 November 1995 (27.11.95)      EP <b>(34) Countries for which the regional or international application was filed:</b> NL et al. 60/007,564                      27 November 1995 (27.11.95)      US  <b>(71) Applicants (for all designated States except US):</b> GIST-BROCADES B.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL). UNIVERSITY OF OXFORD [GB/GB]; University Offices, Wellington Square, Oxford OX1 2JD (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SUTHERLAND, John, David [GB/GB]; 1 Bridge Street, Osney Island, Oxford OX2 0BA (GB). BOVENBERG, Roelof, Ary, Lans [NL/NL]; 's-Gravenweg 121, NL-3062 ZD Rotterdam (NL). VAN DER LAAN, Jan, Metske [NL/NL]; Leursebaan 364, NL-4839 AP Breda (NL).			<b>(74) Agents:</b> VISSER-LUIRINK, Gesina et al.; Gist-Brocades N.V., Patents and Trademarks Dept., Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 17 July 1997 (17.07.97)
<b>(54) Title:</b> IMPROVED PROCESS FOR THE PRODUCTION OF SEMI-SYNTHETIC CEPHALOSPORINS VIA EXPANDASE ACTIVITY ON PENICILLIN G			
<b>(57) Abstract</b>  An overall process for the preparation and recovery of 7-aminodesacetoxycephalosporanic acid (7-ADCA) via enzymatic ring expansion activity on penicillin G, using a modified expandase enzyme.			

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# INTERNATIONAL SEARCH REPORT

In .tional Application No  
PCT/EP 96/05358

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/52 C12N9/00 C12P35/00 C12N1/21		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C12P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE 7TH INTERNATIONAL SYMPOSIUM OF THE GENETICS OF INDUSTRIAL MICROORGANISMS, 1994, page 184 XP000646621 BALDWIN JE ET AL.: "Genetic engineering of cephalosporin biosynthesis" cited in the application see abstract P.262 <div style="text-align: center; margin-top: 10px;">           ---            -/--         </div>	1-9
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
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Date of the actual completion of the international search  <div style="text-align: center; font-size: 1.2em;">27 May 1997</div>		Date of mailing of the international search report  <div style="text-align: center; font-size: 1.2em;">03.06.97</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer  <div style="text-align: center; font-size: 1.2em;">Espen, J</div>

# INTERNATIONAL SEARCH REPORT

I. International Application No

PCT/EP 96/05358

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>CURRENT GENETICS, vol. 17, 1990, pages 213-221, XP000606288 CANTWELL C A ET AL: "CLONING AND EXPRESSION OF A HYBRID STREPTOMYCES CLAVULIGERUS CEFE GENE IN PENICILLIUM CHRYSOGENUM" see page 221, left-hand column, last paragraph</p> <p>---</p>	1-9
E	<p>WO 96 38580 A (GIST BROCADES BV ;BOVENBERG ROELOF ARY LANS (NL); KOEKMAN BERTUS P) 5 December 1996 see the whole document</p> <p>---</p>	1-9
A	<p>ENZYME AND MICROBIAL TECHNOLOGY, vol. 17, 1995, pages 231-234, XP000646836 MAEDA K ET AL.: "The substrate specificity of deacetoxycephalosporin C synthase ('expandase') of Streptomyces clavuligerus is extremely narrow" cited in the application</p> <p>-----</p>	

### Information on patent family members

PCT/EP 96/05358

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